Hammerhead Ribozyme-Mediated Sensitization of Human Tumor Cells after Treatment with 1,3-Bis(2-chloroethyl)-1-nitrosourea

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ABSTRACT

O6-Methylguanine DNA methyltransferase (MGMT) protects tumor cells from the cytotoxic effects of DNA-alkylating agents such as 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU). To improve the therapeutic index of BCNU, biochemical strategies to inhibit MGMT temporarily by systemic administration of small molecules, such as O6-benzylguanine, have been developed and are showing promise in clinical trials. In this study, an alternative molecular strategy for modulating BCNU resistance was explored using hammerhead ribozymes (Rz) designed to degrade the long-lived MGMT mRNA. We had previously identified several ribozymes capable of decreasing MGMT levels in HeLa cells. Using colony formation assays, the BCNU-induced cell kill was shown to be increased by 1 to 3 logs in the HeLa/Rz clones compared with wild-type HeLa cells at a BCNU dose of 100 μM. In the current study, 10 randomly selected clones of Rz161, 212, and a reconstructed Rz178/212 were assayed for MGMT activity, MGMT mRNA, and sensitivity to BCNU. The 30 clones exhibited almost identical results in the three assays, i.e., nearly undetectable MGMT activity, greatly diminished MGMT mRNA, and comparable sensitivity to BCNU using the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) viability assay. The effects of catalytically inactive ribozymes carrying a single point mutation were compared with their active counterparts in vitro and in stably transfected clones to determine whether antisense inhibition was a contributor to the inhibition of MGMT activity we observed. Collectively, these results suggest that the hammerhead ribozymes characterized in this study will be excellent candidates for future gene therapy approaches targeting MGMT.

Chloroethylnitrosoureas (CENUs), herein represented by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), are clinically useful cancer chemotherapeutic agents. However, their antineoplastic activities against human tumors are much lower than their activity against animal tumors (Carter and Wasserman, 1976). The enzyme O6-methylguanine DNA-methyltransferase (MGMT) is expressed in more than 80% of human tumor cell lines and plays a major role in CENU resistance (Erickson, 1991; Mitra and Kaina, 1993). MGMT prevents the generation of the cytotoxic lesion, the DNA interstrand cross-link between guanine and cytosine induced by the CENU, by stoichiometrically removing the chloroethyl monoaduct from the O6-position of guanine through a suicidal transfer to the active site cysteine thiol. The replacement of inactivated MGMT requires de novo protein synthesis (Kroes and Erickson, 1995). Biochemical strategies have been developed to deplete MGMT by pretreatment of tumor cells with alkylating agents, such as streptozotocin (Futscher et al., 1989), or alkylguanine analogs, such as O6-benzylguanine (Dolan et al., 1991). These inhibitors make innately CENU-resistant tumor cells sensitive. However, biochemical strategies are not selective for tumor cells over normal cells, and they inhibit MGMT only temporarily. The de novo synthesized MGMT proteins take the place of inactivated ones and are responsible for the gradual recovery of MGMT activity and resistance to CENU in the tumor cells by 24 h after pretreatment with biochemical inhibitors to deplete MGMT (Kroes and Erickson, 1995).

In the present study, an alternative strategy was exploited to deplete the MGMT system in tumor cells by inhibiting
Hammerhead Ribozymes Able to Deplete MGMT in Tumor Cells

MGMT de novo protein synthesis using hammerhead ribozymes designed to degrade the long-lived MGMT mRNA. A ribozyme is a single RNA molecule containing all of the RNA-encoded functions that can catalyze the cleavage of the phosphodiester backbone of a target mRNA (Cech, 1987; Haseloff et al., 1988). Because the hammerhead ribozymes can recognize and bind with the specific RNA substrate by antisense hybridization and subsequently cleave the mRNA at unique GUC triplet sites, they can be designed to downregulate gene expression in a sequence-specific manner (Bratty et al., 1993; Grassi and Marini, 1996; Birikh et al., 1997; Curcio et al., 1997). One of the major problems in directing the destruction of mRNA by ribozymes is the selection of the proper target site(s). We have demonstrated previously (Zhang et al., 2001), using pooled ribozymes against multiple GUC triplets in HeLa cells, the selection of optimum ribozyme target site(s) within the MGMT mRNA. The loss of the CENU resistant phenotype in the tumor cells was investigated by identification of tumor cell clones sensitized to BCNU. Three ribozyme target sites within the MGMT mRNA, nucleotides 161, 178, and 212 (according to the MGMT mRNA sequence; Tano et al., 1990), with medium-to-high ribozyme sensitivity were identified. After polymerase chain reaction amplification and sequencing, we observed that one of the most BCNU-sensitive clones, HeLa/Rz212/178, contained two ribozyme constructs against nucleotides 178 and 212 in the 5′ region of MGMT mRNA. However, two HeLa/Rz clones containing Rz178 alone showed only modest reductions in MGMT levels in this study. It is therefore important to determine whether the significantly greater level of MGMT reduction in HeLa/Rz212/178 is due to an additive effect of Rz212 and Rz178 or the activity of Rz212 alone.

In this study, the cleavage activity of active ribozymes was demonstrated by the in vitro ribozyme-mediated cleavage of MGMT mRNA. Stably transfected HeLa cell clones were picked randomly to investigate cellular CENU sensitivity, MGMT activity, MGMT protein level, and mRNA expression. The optimum activity of each potential active ribozyme was confirmed and characterized individually.

It is not completely clear that ribozyme-mediated inhibition of gene expression is due to the cleavage activity of the ribozyme, its inherent antisense activity, or both. To determine whether the ribozyme activity is due to the cleavage or antisense binding at the target site(s), both the catalytic cleavage activity and antisense inhibition of gene expression by ribozymes were investigated in this study. To assess the contribution of the antisense effect of ribozymes on the inhibition of MGMT gene expression, mutant ribozymes that are catalytically inactive were used. A single point mutation was made within the conserved core sequence essential for cleavage in each identified active ribozyme. The sequence for the antisense hybridization with the MGMT mRNA was kept intact. Thus, these mutant ribozymes (InacRz) can bind to MGMT mRNA in an antisense manner, while losing their cleavage activities. The mutant ribozymes were introduced into HeLa cells by the same delivery method. The effect of these pure antisense ribozymes was evaluated by the assays for BCNU sensitivity, MGMT activity, MGMT protein, and mRNA in the HeLa/InacRz clones.

Materials and Methods

Materials. BCNU (Carmustine) was purchased from Sigma-Aldrich (St. Louis, MO) and stored at −20°C. All chemical solutions used for RNA preparation and analysis were either treated with diethyl pyrocarbonate or dissolved in 0.2% diethyl pyrocarbonate-treated water. Unless otherwise stated, other chemical reagents were obtained from Sigma-Aldrich, Fisher Scientific (Chicago, IL), and Invitrogen (Carlsbad, CA).

Cell Culture. HeLa (MGMT+*) and HeLa MR (MGMT−) cells were cultured in α-minimum essential medium (Invitrogen) supplemented with 10% bovine calf serum (Hyclone Laboratories, Logan, UT), 1% glutamine, 1% HEPES, and 2% penicillin-streptomycin (all from Invitrogen) and maintained at 37°C in a humidified 5% CO2 atmosphere.

Exogenous Delivery Construction of Active and Mutant Ribozymes. The cDNA sequence of the ribozymes used in this study was as follows: Rz161, 5′-TCGAAACCACTGTAGGTCCGAAAAGGACGAAACAGCTCAG-3′; and Rz212, 5′-TCGAAAGCTGTTCGACGATGAGTCCTGCAAGGACGAAACAGCTCAG-3′; Three point mutant ribozymes InacRz161, InacRz178, and InacRz212 were designed, respectively, to have the identical antisense hybridization arms with their catalytically active counterparts, Rz161, Rz178, and Rz212, while carrying a single point mutation within the conserved catalytic core sequence (U29A). The cDNA sense sequences of these catalytic inactive ribozymes, with point mutation underscored, are as follows: InacRz161, 5′-TCGAAACCACTGTAGGTCCGAAAAGGACGAAACAGCTCAG-3′; InacRz178, 5′-TCGAAAGCTGTTCGACGATGAGTCCTGCAAGGACGAAACAGCTCAG-3′; and InacRz212, 5′-TCGAAAGCTGTTCGACGATGAGTCCTGCAAGGACGAAACAGCTCAG-3′. Three complementary single-stranded oligodeoxynucleotides containing the sequence encoding each of the active and mutant ribozymes were synthesized by Invitrogen. These oligonucleotides, when annealed, possessed cohesive ends to allow directional subcloning into the pcDNA3.1(−)/Hygro mammalian expression vectors (Invitrogen) between the XhoI and HindIII restriction sites. Plasmid DNA was purified by anion exchange chromatography (Qiagen, Valencia, CA).

Stable Transfection of HeLa Cells. Each of the three active and inactive ribozyme expression plasmids was individually transfected into HeLa cells using the LipofectAMINE-PLUS reagent (Invitrogen). The Zeocin-resistant clones for each catalytically inactive ribozyme were selected by 400 μg/ml Zeocin and expanded in complete medium containing 100 μg/ml Zeocin. The cDNA sequence of the ribozymes used in this study

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Cell Survival 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzenedi sulfonate (WST-1) Assay. To screen for the BCNU-sensitive HeLa/Rz clones, the WST-1 (Roche Diagnostics, Mannheim, Germany) cell proliferation assay was used as described previously (Zhang et al., 2001).

Colony Formation Assay. Cells (2.5 × 104) were seeded in 20 ml of complete medium in 75-cm2 flasks and incubated in medium without antibiotics under standard conditions for 24 h. Various doses of BCNU (12.5, 25.0, 50, 75, and 100 μM) or the same volume of 95% ethanol (less than 0.5% final volume) were separately added to each
flask, and cells were treated for 1 h. The cells were then washed with phosphate-buffered saline (PBS, Invitrogen), trypsinized, counted, with a Coulter counter (Beckman Coulter Inc., Miami, FL), and seeded into triplicate 100-mm cell culture dishes (Falcon, Franklin Lakes, NJ) at densities of 300, 500, 1000, 3000, and 10,000 cells/dish in complete medium. After incubation under standard conditions for 15 days, colonies were fixed with methanol, stained with 10% methylene blue in PBS, and counted. Survival fraction is represented by the percentage of the visible colonies formed in the BNCU treated cultures and compared with the control colonies treated with vehicle alone. Survival curves depict the mean and standard deviation of replicates from three independent experiments.

**MGMT Functional Assay.** MGMT activity was measured as described previously (Krekla et al., 1999, 2001). The MGMT substrate consisted of a fluorometric (5'-hexachlorofluorescein (HEX) phosphoramidite-labeled), double-stranded 18-bp oligonucleotide containing a single O6-methylguanine residue nested within a PuVII restriction site (Genosys Biotechnologies, Inc., The Woodlands, TX), which yielded a 10-bp, labeled PuVII cleavage product. The HEX-labeled 18- and 10-bp fragments were detected using a Hitachi FMBIO II fluorescence imaging system (Hitachi Genetic Systems, South San Francisco, CA), and the fluorescence was quantitated using FMBIO Analysis software (Hitachi Genetic Systems). Cellular MGMT activity is directly proportional to the substrate cleavage and is thus represented as the percentage of the 10-bp fragment fluorescence relative to the total amount of substrate fluorescence (10 bp + 18 bp):

\[
\text{Percentage of cleavage} = \left( \frac{\text{fluorescent units of 10mer fragment}}{\text{fluorescent units of 18mer + 10mer fragment}} \right) \times 100\%
\]

**Western Blot Analysis.** Western blots were performed with 50 μg of cellular protein as described previously (Zhang et al., 2001). To assure equal loading of samples, a second primary antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Biodesign International, Saco, ME) was used.

**Northern Blot Analysis.** Northern analysis was performed with 20 μg of total cellular RNA as described previously (Zhang et al., 2001). The Northern blot was quantitated using a Cyclone PhosphorImager (PerkinElmer Life Sciences, Boston, MA) and analyzed using the manufacturer's software.

**Design of Ribozyme and MGMT RNA Templates for In vitro Transcription.** The templates for in vitro transcription with a T7 priming site for ribozymes Rz161, Rz178, and Rz212 and mutant ribozymes InaRz161, InaRz178, and InaRz212 were designed and synthesized (Invitrogen). The antisense sequences for in vitro transcription are as follows (the underlined sequences are T7 priming sites): Rz161, 5'-cttgacagtcttggctccttcgactcatactggttgcctataggtgtgttac-3'; Rz178, 5'-tgcagacagttgctcgcttcgactcatactggttgcctataggtgtgttac-3'; Rz212, 5'-agggacgtgctttgccttcgactcatactggttgcctataggtgtgttac-3'; InaRz161, 5'-cttgacagtcttggctccttcgactcatactggttgcctataggtgtgttac-3'; InaRz178, 5'-tgcagacagttgctcgcttcgactcatactggttgcctataggtgtgttac-3'; and InaRz212, 5'-agggacgtgctttgccttcgactcatactggttgcctataggtgtgttac-3'. The template for in vitro transcription of a target MGMT mRNA fragment, from nucleotide 148 to 224, including the three potential cleavage sites of nucleotide 161, 178, and 212, primed by a T7 site, was designed and synthesized (Invitrogen).

**MGMT Target mRNA Fragment (nt 137-237).** 5' gcagacagtctcacttcgactcatactggttgcctataggtgtgttac-3'; 3' T7 sense primer for annealing 5' tataagcagactcatactggttgcctataggtgtgttac-3'.

**In Vitro Transcription of Ribozyme and MGMT RNA Fragments.** T7 sense primer oligonucleotides and antisense templates for ribozymes, mutant ribozymes, or MGMT mRNA fragments were suspended in TES (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.1 M NaCl) buffer. Equimolar amounts of T7 primer and template were annealed. The T7-MEGAscript high-yield transcription kit (Ambion, Austin, TX) was used according to the manufacturer’s instructions. Briefly, 2 μl of 10× transcription buffer, 2 μl of 75 mM ATP solution, 2 μl of 75 mM UTP solution, 2 μl of 75 mM GTP solution, 2 μl of 75 mM CTP solution, 1 μl of 3.3 mM [α-32P]CTP (3000 Ci/mmole, 10 μCi/ml; PerkinElmer Life Sciences), and annealed template DNA (template final concentration of 300 and 100 nM for ribozyme and MGMT mRNA fragments, respectively) were added sequentially into an RNase-free Microfuge tube and mixed thoroughly, and then 2 μl of T7 MEGAscript enzyme mix was applied and the final volume was adjusted to 20 μl with RNase-free distilled H2O. The reaction was incubated in a 37°C incubator for 6 h. After incubation, 1 μl of RNase-free DNase (2 U/μl) was added to reaction, mixed thoroughly, and incubated at 37°C for 15 min. The reaction was terminated by adding 20 μl of formamide gel loading buffer, vortexing, and heating at 95°C for 5 min and stored at -20°C. In the in vitro transcription products were separated by 20% PAGE at 26 mA for 4 h. The gel was visualized by autoradiography. The band of proper transcribed product was cut out by aligning the film with the gel, and the excised band was transferred to an RNase-free tube and crushed in 5 μl of RNase-free TE buffer. The tightly capped tube was then vortexed at 20°C for 2 h. After spinning at 1000 rpm for 5 min, the acrylamide elute was transferred to an RNase-free tube and made 0.3 M in sodium acetate, pH 5.2, and 2 μg of molecular biology grade glycogen was added (Roche Diagnostics, Basel, Switzerland), mixed briefly, and finally precipitated by the addition of 3 volumes of ice-cold ethanol. The washed, dried pellet was resuspended in 100 μl of RNase-free 50 mM Tris-HCl and stored at -80°C until use.

**Results**

**Ribozyme-Mediated in Vitro Cleavage of MGMT RNA.** To determine whether the ribozymes were functional in vitro, ribozyme and target RNA were mixed and incubated at different molar ratios at 60°C in the presence of MgCl2. Figure 1 demonstrates the formation of expected cleavage products by the active ribozymes at 3:1 and 6:1 M ratio (Rz:MGMT RNA) at 60°C in a 10-min cleavage reaction. MGMT target (77 nt) RNA was cleaved by the three ribozymes (39nt) at each of the three specific sites to generate products by the active ribozymes at 3:1 and 6:1 M ratio) (Rz:MGMT RNA) at 60°C in a 10-min cleavage reaction. MGMT target (77 nt) RNA was cleaved by the three ribozymes (39nt) at each of the three specific sites to generate products by the active ribozymes at 3:1 and 6:1 M ratio) MGMT target RNA was cleaved by Rz212, and over 90% of the target was cleaved by Rz161 and Rz178 at either 3:1 or 6:1 M ratio.

**WST-1 Cell Proliferation Assay to Measure BCNU Sensitivity in Reconstituted HeLa/Rz Clones.** Ten stably transfected cell clones of HeLa/Rz161, HeLa/Rz212, and...
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Fig. 1. Ribozyme-mediated in vitro cleavage of MGMT RNA. Autoradiograph of 10% denatured PAGE showing the ribozyme-mediated in vitro cleavage of MGMT RNA at 3:1 and 6:1 M ratio (Rz: MGMT RNA) at 60°C with the presence of 10 mM MgCl₂. MGMT target (77nt) was cleaved by three ribozymes (39nt) at three specific sites to generate cleaved fragments of 65, 63, and 46 nt, for Rz212, Rz178, and Rz161, respectively.

HeLa/RzH212/Z178 were analyzed using triplicate short-term WST-1 cell proliferation assay with treatment of a sublethal dose of 100 μM BCNU, respectively (Table 1). The wild-type HeLa cells, which are methylation repair positive (Mer⁺) with high MGMT activity, were shown to be resistant to a sublethal dose of 100 μM BCNU; the mean value of cell viability of HeLa cells was 91%. HeLa MR cells, which are methylation repair negative (Mer⁻) and MGMT deficient with no detectable MGMT activity, were highly sensitive to 100 μM BCNU. The mean value of cell viability of HeLa MR was 38%. All of 10 HeLa/Rz161 clones showed obvious BCNU sensitivity compared with wild-type HeLa cells, with cell viability varying from 31 to 56% and mean of 39% (Table 1). All of 10 HeLa/Rz212 clones showed significant BCNU sensitivity compared with wild-type HeLa cells, with cell viability varying from 40 and 51% and mean of 43% (Table 1, middle column grouping). All of 10 HeLa/Rz161 clones showed to be sensitive to the sublethal dose of 100 μM BCNU, with variable cell viability from 33 to 44% and mean of 40% (Table 1, right column grouping). The percentage of cell viability between HeLa/Rz clones and wild-type HeLa cells was statistically significant at \( p < 0.05 \) when compared using Student’s t test, whereas cellular viability among different HeLa/Rz clones was not statistically significant. One HeLa/Rz clone with BCNU sensitivity closest to the mean value within each group of HeLa/Rz161, HeLa/Rz212, and HeLa/RzH212/Z178 clones was picked and expanded for further characterization. These three HeLa/Rz clones were named as HeLa/Rz161, HeLa/Rz212, and HeLa/H212/Z178, respectively.

MGMT Functional Assay to Measure MGMT Activity in Cell Extract from Reconstituted HeLa/Rz Clones. MGMT activity in cell extracts was compared among wild-type HeLa, HeLa MR, and HeLa/Rz161, HeLa/Rz212, and HeLa/RzH212/Z178 clones (Fig. 2). Wild-type HeLa cells exhibited the high-level MGMT activity, generating about 59% substrate cleavage. Under the same conditions, MGMT activity was undetectable in the HeLa MR cells. HeLa/Rz161, HeLa/Rz212, and HeLa/RzH212/Z178 all showed a low level of MGMT activity with 8, 2.5, and 4.2% substrate cleavage. The reduction of MGMT activity in HeLa/Rz161, HeLa/Rz212, and HeLa/RzH212/Z178 clones were statistically significant compared with wild-type HeLa cells (\( p < 0.05 \)).

Western Blot Analysis of MGMT Protein in Reconstituted HeLa/Rz Clones. As shown in Fig. 3, robust levels of MGMT protein were found in cell extracts from wild-type HeLa cells. MGMT protein was not detected in HeLa MR cells, which lack \( O^{6} \)-alkylation repair activity. MGMT levels were dramatically decreased and barely detected by Western blot analysis in HeLa/Rz161, HeLa/Rz212, and HeLa/RzH212/Z178 clones compared with wild-type HeLa cells.

Northern Blot Analysis of Total RNA in Reconstituted HeLa/Rz Clones. A high level of MGMT mRNA was detected in wild-type HeLa cells by Northern analysis (Fig. 4). No MGMT mRNA was detected in HeLa MR cells. This was consistent with the reports that MGMT mRNA is absent in spite of the presence of the MGMT gene in most Mer⁻ and CENU-sensitive human tumor cells, e.g., HeLa MR cells. MGMT mRNA levels were dramatically decreased by 89, 93, and 85% in HeLa/Rz161, HeLa/Rz212, and HeLa/RzH212/Z178 clones, respectively, compared with the wild-type HeLa cells.

No in vitro Cleavage of MGMT RNA Catalyzed by Mutant Ribozymes. To determine whether the mutant ribozymes were catalytically functional in vitro, the three mutant ribozymes and target RNA were mixed in separate reactions and incubated at a 3:1 M ratio at 60°C in the presence of cell viability between HeLa/Rz clones and wild-type HeLa cells was statistically significant at \( p < 0.05 \) when compared using Student’s t test, whereas cellular viability among different HeLa/Rz clones was not statistically significant. One HeLa/Rz clone with BCNU sensitivity closest to the mean value within each group of HeLa/Rz161, HeLa/Rz212, and HeLa/RzH212/Z178 clones was picked and expanded for further characterization. These three HeLa/Rz clones were named as HeLa/Rz161, HeLa/Rz212, and HeLa/H212/Z178, respectively.

### Table 1

<table>
<thead>
<tr>
<th>Clones</th>
<th>Viability (mean ± S.D.)</th>
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<tbody>
<tr>
<td>HeLa</td>
<td>91.2 ± 8.5</td>
</tr>
<tr>
<td>HeLa MR</td>
<td>37.7 ± 3.7</td>
</tr>
<tr>
<td>Clone 1</td>
<td>40.7 ± 4.7</td>
</tr>
<tr>
<td>Clone 2</td>
<td>31.3 ± 3.2*</td>
</tr>
<tr>
<td>Clone 3</td>
<td>36.5 ± 4.9</td>
</tr>
<tr>
<td>Clone 4</td>
<td>35.0 ± 11.2</td>
</tr>
<tr>
<td>Clone 5</td>
<td>36.3 ± 2.1</td>
</tr>
<tr>
<td>Clone 6</td>
<td>39.7 ± 3.2</td>
</tr>
<tr>
<td>Clone 7</td>
<td>55.6 ± 4.5</td>
</tr>
<tr>
<td>Clone 8</td>
<td>39.3 ± 4.2</td>
</tr>
<tr>
<td>Clone 9</td>
<td>34.0 ± 3.6*</td>
</tr>
<tr>
<td>Clone 10</td>
<td>39.3 ± 2.1</td>
</tr>
</tbody>
</table>

All changes in cell viabilities were significant (\( P < 0.05 \)), and those denoted with an asterisk (*) had \( P < 0.005 \).
of MgCl₂, the identical conditions for the active ribozyme-mediated in vitro cleavage reactions. Figure 5 shows the autoradiograph of the 10% denatured PAGE. It was demonstrated that there was no cleavage of the MGMT target RNA observed with any of the three mutant ribozymes and substrates at 3:1 M ratio. However, all three active ribozymes catalyzed significant cleavage at their designated sites under the same conditions. The three mutant, catalytically inactive ribozymes were thereafter named as InacRz161, InacRz178, and InacRz212.

BCNU Sensitivity in Stably Transfected HeLa/InacRz Clones. In the triplicate WST-1 cell proliferation assay (Table 2), wild-type HeLa cells, HeLa MR cells, and 10 clones of HeLa/InacRz161, HeLa/InacRz178, and HeLa/InacRz212 were measured for BCNU sensitivity. The Mer⁻ HeLa cells were shown to be resistant to a sublethal dose of 100 μM BCNU. In contrast, HeLa MR cells, which are Mer⁻ and MGMT-deficient with no detectable MGMT activity, were highly sensitive to 100 μM BCNU. As shown in Table 2, all of the 10 HeLa/InacRz161 clones demonstrated a high degree of resistance to BCNU, where cell viability varied from 83 to 93% with a mean of 90%. Similarly, all 10 HeLa/InacRz178 clones were shown to be resistant to the sublethal dose of BCNU; cell viability of each clone varied between 78 and 94%, with a mean of 87%. Finally, all 10 HeLa/InacRz212 clones were demonstrated to be BCNU-resistant, with cell viability varying between 82 and 92%, with a mean of 88%. The difference in cellular viability among the group of HeLa/InacRz clones was not statistically significant. There is also no statistically significant difference between cell viability of wild-type HeLa cells and that of HeLa/InacRz161, 178, or 212 clones. One HeLa/InacRz clone closest to the mean value of cell viability in each group was therefore picked and expanded for further characterization.

MGMT Functional Assay to Measure MGMT Activity in Cellular Extract of HeLa/InacRz Clones. Wild-type HeLa cells exhibited considerable MGMT activity, generating about 60% substrate cleavage (Fig. 6, A and B). There was no MGMT activity detected in the HeLa MR cells. The HeLa/Rz161 and HeLa/Rz212 clones with active ribozymes showed a low level of MGMT activity with 8 and 5% probe cleavage, respectively. Conversely, HeLa/InacRz161 and HeLa/InacRz212 clones, with ribozymes containing the A29U point mutation, showed high MGMT activity with 58 and 65% probe cleavage under the same condition. HeLa/Rz178 and HeLa/InacRz178 showed 54 and 63% probe cleavage, respectively. These results underscore the reproducibility of our observations that Rz178 was only modestly effective in reducing MGMT activity in these cells. The reduction of MGMT activity in HeLa/Rz161 and HeLa/Rz212 clones was statistically significant (P < 0.05), compared to HeLa/InacRz161 and HeLa/Inac212 clones.

Western Blot Analysis to Measure Cellular MGMT Protein in HeLa/InacRz Clones. To further characterize MGMT protein levels in these stably transfected clones, Western blot analysis was performed. As shown in Fig. 7, MGMT protein was readily detected in cell extract from wild-type HeLa cells and was undetectable in HeLa MR cells. Essentially wild-type levels of MGMT protein were found in HeLa/InacRz161 and HeLa/InacRz212 clones, whereas MGMT protein levels were decreased more than 80% in their active counterparts, the HeLa/Rz161 and HeLa/Rz212

Fig. 2. MGMT functional assay to measure MGMT activity in cell extracts. Activity was measured by incubating 50 μg of cellular extract with an 18mer fluorometric HEX-labeled oligonucleotide substrate for MGMT, digested with PvuII, and run on a 20% denaturing PAGE. Percent cleavage = fluorescence of 10mer/(10mer + 18mer), which is directly proportional to MGMT activity in the cellular extract. Data represent mean ± S.D. from three independent experiments.

Fig. 3. Western blot analysis to measure cellular MGMT protein level. Cellular protein (30 μg) and monoclonal hMGMT antibody were used to measure MGMT protein levels in wild-type HeLa, HeLa MR, and HeLa/ Rz161, HeLa/Rz212, and HeLa/RzH212/Z178 clones. The filter was reprobed for GAPDH protein levels to demonstrate equivalent loading.

Fig. 4. Northern blot analysis of total RNA using hMGMT cDNA probe. Total mRNA (20 μg) from wild-type HeLa cells, HeLa MR cells, and HeLa/Rz161, 212, and H212/Z178, was used, respectively, to determine MGMT mRNA levels. The blot was reprobed for β-actin mRNA to demonstrate equivalent loading.
clones. HeLa/InacRz178 and HeLa/Rz178 clones both showed a high level of MGMT protein. The housekeeping protein GAPDH confirmed equal loading of these samples.

**Northern Blot Analysis of Total RNA in HeLa/InacRz Clones.** As shown in Fig. 8, abundant MGMT mRNA was detected in wild-type HeLa cells. No MGMT mRNA was detected in HeLa MR cells. MGMT mRNA levels detected in HeLa/InacRz161, 178, and 212 clones were little different from that of wild-type HeLa cells; whereas significantly reduced and barely detectable levels of MGMT mRNA existed in the HeLa/Rz161, HeLa/Rz212, and HeLa/RzH212/Z178 clones, where constitutively produced, active ribozyme was introduced.

**BCNU Sensitivity in HeLa/Rz and HeLa/InacRz Clones Determined Quantitatively by Colony Formation Assay.** BCNU sensitivity, regulated by catalytically active and inactive ribozymes in HeLa/Rz and HeLa/InacRz clones, respectively, was quantitatively determined by colony formation assay. In Fig. 9, colony formation assays showed wild-type HeLa cells were resistant to BCNU at the sublethal dose of 100 μM. HeLa MR, on the other hand, was shown to be highly sensitive to BCNU treatment at this dose. The BCNU-induced cell kill was dramatically increased by 2 to 3 logs in HeLa/Rz161, HeLa/Rz212, and HeLa/RzH212/Z178 clones, compared with wild-type HeLa cells, after a 1-h exposure to 100 μM BCNU, which was consistent with the colony formation assays of the initially identified HeLa/Rz clones (Zhang et al., 2001). The cell survival fractions of HeLa/Rz161, 212, and H212/Z178 were similar to that of HeLa MR cells, showing exquisite BCNU sensitivity. Conversely, the HeLa/InacRz161, 178, and 212 clones, which express catalytically inactive ribozymes, show little or no increase of BCNU-induced cell kill under the same condition, which was not statistically significant compared with that of wild-type HeLa cells.

### Discussion

CENUs, represented in this work by BCNU, are clinically useful cancer chemotherapeutic agents. However, their antineoplastic activities against human tumors are much less effective than their activity against animal tumors (Carter and Wasserman, 1976). The enzyme MGMT is expressed in more than 80% of human tumor cell lines in culture and plays...
the major role in cellular CENU resistance (Erickson, 1991; Mitra and Kaina, 1993). Several biochemical strategies have been developed to deplete MGMT by pretreatment of tumor cells with alkylating agents, such as temozolomide (Kokki-nakis et al., 2001) or alkylguanine analogs, such as O⁶-methylguanine (Dolan et al., 1989) or O⁶-benzylguanine (Dolan et al., 1991).

In our previous publication (Zhang et al., 2001), an alternative strategy to deplete MGMT activity in tumor cells was investigated and developed using pooled hammerhead ribozymes. This strategy inhibits MGMT de novo protein synthesis by using chimeric hammerhead ribozymes designed to hybridize to and degrade the long-lived MGMT mRNA. Three potential target sites, nucleotides 161, 178, and 212, within MGMT mRNA were identified. It was noticed after polymerase chain reaction amplification and sequencing, that one of the representative HeLa/Rz161, HeLa/Rz212, and HeLa/RzH212/178 clones. It was notable that the HeLa/Rz212 clone containing Rz212 alone showed significant BCNU sensitivity with dramatically low levels of MGMT activity. This BCNU sensitivity was similar to that of the dual-ribozyme expressing clones HeLa/Rz212/178 and the reconstituted HeLa/RzH212/178. Thus, Rz212 alone was identified as having significant activity in depleting MGMT activity.

To investigate the catalytic cleavage activity of hammerhead ribozymes at unique target sites within the MGMT mRNA, in vitro ribozyme-mediated cleavage reactions were performed. The presence of Mg²⁺ in the ribozyme-mediated in vitro cleavage reaction was essential for hammerhead ribozyme catalysis. The metal ion plays two roles: it promotes proper folding of the RNA to form the catalytic core and it acts as a catalytic cofactor (Dahm and Uhlenbeck, 1991). The efficiency of hammerhead ribozymes in cleaving an RNA transcript in vitro has been shown to be correlated with ribozyme-mediated gene regulation in vivo (Sun et al., 1994). A ribozyme that failed to cleave the transcript in vitro did not show any inhibition in cell culture (Lieber and Strauss, 1995). However, sometimes, the in vitro results are not completely consistent with the performance of a ribozyme in vivo, because there are many factors in the cell, such as low free Mg²⁺ concentrations, the stability of the ribozyme, and compartmentalization of the ribozyme and substrate, that may affect the rate of ribozyme-substrate association and catalytic cleavage (Birikh et al., 1997). Some RNA binding proteins and nuclear proteins might be involved in the ribozyme-catalyzed cleavage (Tsuchihashi et al., 1993; Heidenreich et al., 1995). Some proteins might facilitate ribozyme turnover by catalyzing the annealing of the RNA substrate to the ribozyme and the dissociation of cleaved products; on the other hand, some proteins may inhibit the ribozyme’s activity by competing for the same binding site within the target mRNA (L’Huillier et al., 1992; Herschlag et al., 1994; Birikh et al., 1997).

It is known that by complementary binding to the specific sequence of the target mRNA, antisense RNA can inhibit translation without cleaving the RNA, thus down-regulating the synthesis of a specific gene product (Scanlon et al., 1995). On the other hand, after binding with the mRNA substrate, the ribozyme can catalyze the cleavage reaction at specific GUC triplet sites. To characterize the molecular mechanism of these ribozymes, investigations with catalytically inactive ribozymes InacRz161, InacRz178, and InacRz212, whose active counterparts were identified, were studied. The point mutation of A29U made in the ribozyme catalytic core sequence is known to abolish ribozyme catalytic activity by improper folding of the ribozyme secondary structure, while
keeping its antisense hybridization capability (Ruffner et al., 1990; Woisard et al., 1994; Domi et al., 1996). These point mutant ribozymes were shown to be incapable of cleaving synthetic MGMT mRNA in vitro. The function of mutant ribozymes was evaluated using the assays for BCNU sensitivity, MGMT activity, MGMT protein, and mRNA in the HeLa/InacRz clones. There was no significant MGMT depletion identified with the introduction of these catalytically inactive ribozymes in HeLa cells. Although we did not directly test the ability of these point mutant ribozymes to bind their targets per se, these results strongly suggest that the major mechanism of ribozyme activity we observed is due to the catalytic cleavage of target mRNA, and not to antisense binding to the MGMT mRNA.

The use of hammerhead ribozymes as modulators of specific gene expression has been investigated widely. The most active area of investigation into the application of ribozymes has been the development of gene therapy for cancer and AIDS. Potter et al. (1993) have previously demonstrated the ability of a hammerhead ribozyme targeting position 161 to cleave MGMT mRNA. However, these authors did not report the ability of the ribozyme to sensitize cells to agents such as BCNU. Citti et al. (1999) have shown that transiently transfected, synthetic hammerhead ribozymes could potentiate the genotoxicity of the alkylation damage induced by mitozolomide. This same group has shown that coadministration of O6-methylguanine and a chemically modified hammerhead ribozyme could hypersensitize cells expressing a high level of MGMT to mitozolomide (Citti et al., 1998). These authors concluded that a dual strategy targeting both mRNA and protein holds promise for successfully down-regulating the MGMT gene.

Some of the limiting features of ribozymes in the develop-
ment of gene therapy strategies have been the absence of effective delivery methods and RNA instability. Although the latter can be overcome by the incorporation of chemically modified nucleotides, a technique that is not without its own set of problems, ribozyme delivery remains a challenge. Recently, Aigner et al. (2002) reported the successful delivery of unmodified ribozymes by complexation with low-molecular-weight polyethylenimine. These authors observed inhibition of tumor growth and decreased target gene expression levels in a xenograft mouse model of human melanoma. Thus, this approach, which demonstrated the protection of a number of RNAs, offers the opportunity to effectively deliver unmodified ribozymes in vivo.

The systemic administration of DNA alkylating agents such as carmustine or temozolomide carries with it the downside that normal tissues are also subject to sensitization when treatment would be coupled with an untargeted ribozyme to MGMT. This may lead to undesirable toxicities or levels of DNA damage in certain patients receiving such chemotherapy. The molecular strategy developed here to deplete MGMT using hammerhead ribozymes may be most useful in future gene therapy strategies in which nonspecific toxic effects of several DNA-alkylating agents could be avoided by coupling ribozyme delivery with tumor-specific immunoliposomes, or by using tumor-specific promoters to drive ribozyme expression (Shi and Pardridge, 2000). Future experiments will examine ribozyme expression and MGMT mRNA depletion using an inducible expression system to gain a better understanding of the time course and kinetics of the ribozyme activity we have characterized.

References


